[1] Tough, I. M., K. E. Buckton, A. G. Baikie, and W. M. Court Brown, *Lancet*, **ii**, 849–851 (1960).

[2] Bender, M. A, and P. C. Gooch, *Radiation Research*, **16**, 44–53 (1962).

[3] Boyd, E., W. W. Buchanan, and B. Lennox, *Lancet*, **i**, 977–978 (1961).

[4] Stewart, J. S. S., and A. Sanderson, *Lancet*, **i**, 978–979 (1961).

[5] Conen, P. E., *Lancet*, **ii**, 47 (1961).

[6] Herschberg, S. N., *Lancet*, **ii**, 104 (1961).

[7] Bender, M. A, *Science*, **126**, 974–975 (1957).

[8] Bender, M. A, in *Immediate and Low Level Effects of Ionizing Radiations*, ed. A. A. Buzzati-Traverso (London: Taylor and Francis Ltd., 1960), pp. 103–118.

[9] Puck, T. T., these PROCEEDINGS, **44**, 772–780 (1958).

[10] Chu, E. H. Y., N. H. Giles, and K. Passano, these PROCEEDINGS, **47**, 830–839 (1961).

[11] Lea, D. E., *Actions of Radiations on Living Cells*, 2d ed. (Cambridge: Cambridge University Press, 1955).

[12] Bender, M. A, and S. Wolff, *Am. Naturalist*, **45**, 39–52 (1961).

[13] Bender, M. A, and P. C. Gooch, *Int. J. Radiat. Biol.*, **4**, 175–184 (1961).

[14] Moorhead, P. S., P. C. Nowell, W. J. Mellman, D. M. Battips, and D. A. Hungerford, *Exp. Cell Research*, **20**, 613–616 (1960).

[15] Bender, M. A, and D. M. Prescott, "DNA synthesis and mitosis in cultures of human peripheral leucocytes," *Exp. Cell Research*, in press (1961).

[16] Denver Classification. *Lancet*, **i**, 1063–1065 (1960).

[17] We are indebted to Dr. M. A. Kastenbaum of the Oak Ridge National Laboratory Mathematics Panel for these calculations.

[18] Wolff, S. personal communication (1961).

[19] Fraccaro, M., Discussion of ref. 8, p. 117 (1960).

[20] Lindsten, J., *Uppsala Läkfören. Förh.*, **64**, 8–9 (1959).

[21] Sax, H. J., and K. Passano, *Am. Naturalist*, **45**, 97–102 (1961).

[22] Dubinin, N. P., M. A. Arsen'eva, and Yu. Ya. Kerkis, "Geneticheskaya Opasnost Malykh Doz Radiatsii dlya Cheloveka i ikh Effekt na Nasledstevennost Obezyan i Gryzunov," *Akad. Nauk S.S.S.R. (Moskva)* (1960).

[23] Giles, N. H., "Radiation-induced chromosome aberrations in *Tradescantia*," in *Radiation Biology*, ed. A. Hollaender (New York: McGraw-Hill Book Co., Inc., 1954).

REVERSAL OF MUTANT PHENOTYPES BY 5-FLUOROURACIL: AN APPROACH TO NUCLEOTIDE SEQUENCES IN MESSENGER-RNA

BY SEWELL P. CHAMPE AND SEYMOUR BENZER

DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY

Communicated February 27, 1962

Genetic information in DNA is apparently expressed via transcription into RNA messengers[1–5, 10] which in turn act as the templates for protein synthesis. Thus, incorporation of base analogues into messenger-RNA could lead to errors in the reading of the message into an amino acid sequence. The effect would be an alteration of phenotype without a permanent change in the DNA genotype. A promising analogue for this purpose is 5-fluorouracil (5FU), which is readily incorporated into RNA, mostly in place of uracil.[6] Modification of proteins by 5FU has been reported by Naono and Gros[7] and by Bussard *et al.*[8] who found that the enzymes alkaline phosphatase and β-galactosidase synthesized in the presence of the analogue are abnormal.

A very sensitive method for the detection of induced errors in the translation of

genetic information is to begin with a mutant that is defective in some function so that errors causing the appearance of a small amount of activity can be easily observed. This approach has been successful[9] using *r*II mutants of phage T4, which are ordinarily unable to grow on strain K of *E. coli*. Addition of 5FU after infection of the cell can partially reverse the phage mutant phenotype, leading to active development of the phage. The response is highly specific, occurring only with certain *r*II mutants and not others within the same cistron. Since, after phage infection, synthesis of ribosomal RNA and S-RNA is almost totally arrested,[10] it is plausible that the effect of 5FU on phage mutants is due to its incorporation into messenger-RNA.

Introduction of a fluorine atom at the 5-position in uracil would be expected to induce a positive charge elsewhere in the molecule, increasing the probability of loss of the proton from the 1-position. This would make it possible for 5FU to pair with guanine. Thus, 5FU might produce an effect by either (or both) of two mechanisms: by entering the messenger in place of U and behaving sometimes like C, or by going in (occasionally) like C and later pairing like U. In either case, assuming that the single-stranded messenger-RNA copies the DNA according to the Watson-Crick rules of base pairing, 5FU could reverse the effect of a mutation at a particular DNA site only if the corresponding base in the messenger is a pyrimidine. An additional requirement, if the altered message is to correspond to the original nonmutant one, is that the mutant DNA must have been derived from the standard type by a "transition" mutation,[11] i.e., a substitution in which the orientation of purine and pyrimidine between the two DNA chains remains unchanged.

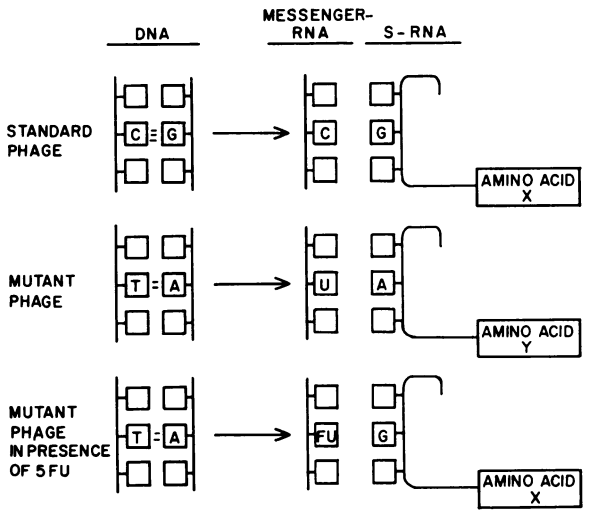

FIG. 1.—Proposed mechanism for the specific action of 5FU. The GC pair in the DNA of the standard (normal) phage is translated as C in the messenger-RNA, and a complementary S-RNA adaptor pairs with the latter, thus specifying amino acid X. For a mutant arising by a GC → AT transition, the base in the messenger-RNA becomes U, specifying an incorrect amino acid Y. In the presence of FU, however, U may be replaced by the analogue, which, when occasionally pairing like C, produces a normal message.

Figure 1 illustrates the proposed mechanism for a mutant that has arisen by substitution, in the phage DNA, of an adenine-thymine (AT) pair for a guanine(hydroxylmethyl) cytosine (GC) pair. At the corresponding site in the messenger-RNA, the mutant has U instead of C, so that the message is incorrect. In the presence of 5FU, however, U may be replaced by the analogue, which, when functioning as C, produces a normal message.

Among mutants containing an AT base pair, the A can be in either strand of the DNA. If each strand of the DNA is transcribed into a functional

messenger, all transition mutants containing AT as the mutant base pair should respond to 5FU. On the other hand, if only one strand of the DNA is transcribed into a useful messenger, about one half of the transition mutants containing AT as the mutant base pair should respond to 5FU.

In this paper, *r*II mutants of phage T4 are analyzed to characterize the mutations in their DNA (by induction of reverse mutations with DNA base analogues) and also for phenotypic response to 5FU. The results indicate that only one functional messenger is produced and suggest the assignment of bases to the various sites in the messenger-RNA.

Materials and Methods.—Bacterial strains: For crosses and for nonselective plating, *E. coli* B was used. *E. coli* BB, which does not discriminate between standard type (r^+) phage and *r*II mutants, was used for growing all phage stocks and as the host for measuring induction of reverse mutations by base analogues. *E. coli* K (the specific variety used here being the KB strain) supports the growth of r^+ but not *r*II mutants, and was used as selective host in detecting reversion from *r*II to r^+. K was also used as the host in testing the 5FU effect on *r*II mutants. *E. coli* K10 and its phosphatase negative derivatives[12, 13] were obtained from Dr. Alan Garen.

rII mutants:[14] All were derived from phage T4B, with the exception of those designated by ED, which are spontaneous mutants derived from T4D. *Spontaneous mutant* numbers have either no prefix, or the prefixes SN or SD. Single-letter prefixes from A through J designate spontaneous mutants derived from revertants of spontaneous *r*II mutants. *Mutagen-induced mutants* are prefixed as follows: NA, NB, or NT induced by nitrous acid; EM by ethyl methane sulfonate; HB by hydroxylamine; N or M by 5-bromouracil or 5-bromodeoxyuridine; AP by 2-aminopurine; DAP by 2,6-diaminopurine; BC by 5-bromodeoxycytidine; P by proflavine; PT, PB by heat at low pH; UV by ultraviolet light. Many of the mutants were contributed by J. Drake, R. Edgar, E. Freese, M. Meselson, and I. Tessman.

Genetic mapping of rII mutants was done by techniques previously described.[14]

Media: Unless otherwise noted, the medium was broth (1% Difco bacto-tryptone plus 0.5% NaCl). For plates, 1.2% agar was added for the bottom layer and 0.7% for the top layer. In experiments involving 5-fluorouracil, a supplemented synthetic medium (M9S) was used containing, per liter of solution, 5.8 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 0.5 gm NaCl, 1.0 gm NH_4Cl, 0.25 gm $MgSO_4 \cdot 7H_2O$, 2.7 mg $FeCl_3 \cdot 6H_2O$, 4.0 gm glucose, 20 mg L-tryptophan, and 2.5 gm Difco vitamin-free casamino acids. M9 is the same medium minus the casamino acids. M9 buffer is the same medium minus the carbon compounds. For experiments involving induction of alkaline phosphatase, Tris-glucose medium was used which contained, per liter, 12.1 gm Tris (Sigma Chemical Co.), 3.0 gm $MgSO_4 \cdot 7H_2O$, 1.0 gm $(NH_4)_2SO_4$, 0.5 gm sodium citrate, 50 mg methionine, and 1.0 gm glucose, pH adjusted to 7.4 with HCl. This medium was used either with 10 gm per liter KH_2PO_4 ("high phosphate") to repress phosphatase synthesis, or with 5 mg per liter ("low phosphate") to induce phosphatase synthesis.[15] *5-fluorouracil* was kindly donated by Dr. R. Duschinsky of the Hoffmann-LaRoche Company.

Reversion induction by base analogues: E. coli BB was grown in M9 to 10^8 cells/ml and the phage in question was added to give about 200 particles per ml. One ml of this mixture was then added to each of three tubes containing, respectively, 1 ml of (*a*) plain M9, (*b*) M9 plus 5-bromodeoxyuridine (0.1 mg/ml) and (*c*) M9 plus 2-aminopurine (1 mg/ml). The tubes were incubated at 37°C for 20 hr, then shaken with a few drops of chloroform to complete lysis. Each lysate was assayed on strain B for total plaque-forming particles and on strain K for revertants; the ratio of the titer on K to the titer on B is the *reversion index*. For all cases in which the reversion index appeared to be raised by a mutagen, the test was repeated at least once to assure that the effect was not due to a "jackpot"[16] (i.e., an abnormally large clone due to early appearance and subsequent replication of a revertant).

Reversion induction with hydroxylamine was tested by the procedure of Freese, Bautz, and Bautz-Freese.[17] One volume of phage stock was added to four volumes of a freshly made reaction mixture containing 1.25 *M* $NH_2OH \cdot HCl$, 1.0 *M* NaCl, 0.001 *M* $MgSO_4$, 0.075 *M* Na_2HPO_4, the pH being adjusted to 7.5 with NaOH. Samples were taken before and after four hours of treatment at 37°C, diluting into a cold reaction-stopping mixture consisting of 0.5% bacto-tryptone,

6% NaCl, and 2% by volume of acetone. Under the conditions used, inactivation of the phage particles by hydroxylamine amounted to roughly 50 per cent. The results are given directly as the reversion index (i.e., ratio of titer on K to titer on B) before and after treatment. This was preferred to expressing them in terms of mutations per lethal hit, the latter quantity being difficult to measure when the killing is so small.

Verification of revertants: For any mutant that was induced to revert a number of plaques (from 4 to 30) was picked from the K plates and replated on strain B as a check on plaque type. In some cases, the plaques were *r* type on B, showing that the "revertants" were false, i.e., did not represent a return to the original standard type. Such false reversions may be due to suppressor mutations at some site other than the original mutation.[18, 19] Therefore, data are reported only for mutants most of whose induced revertants looked genuine on B. This is, of course, a necessary but still not sufficient criterion for genuineness of the revertants.

Assay of phosphatase activity:[20] *p*-nitrophenyl phosphate was dissolved at 5mg/ml in 1 *M* Tris buffer, pH 8.0, and extracted with ether until colorless. This removes traces of *p*-nitrophenol and increases the sensitivity of the assay at low levels of enzyme. The reagent was added to an equal volume of appropriately diluted bacterial culture (previously shaken with a few drops of chloroform) and incubated at 37°C. The rate of color development measured at 410 mμ is a measure of the phosphatase activity.

Results.—Effect of 5FU on rII mutants: Figure 2 shows the effect of 5FU on the activity of various *r*II mutants in *E. coli* strain K. The procedure was to infect K cells with the phage mutant, and dilute into media with or without 5FU. At various times thereafter, the infected cells were further diluted into broth and allowed to lyse. Reversal of the phenotype of an *r*II mutant is reflected in appearance of phage progeny, the yield per infected cell, as compared with that obtained for r^+ phage under the same conditions, being a measure of the degree of activity. While two of the mutants in Figure 2 produce a sizable fraction of the r^+ yield, the other three mutants show practically no response. This striking specificity, which applies to mutants of both cistrons, shows that the effect of 5FU cannot be a general removal of the block against *r*II mutants. Although 5FU is in general somewhat inhibitory, depressing the yield of the standard type phage by about twofold, the increase shown by responsive mutants is over and above this general inhibition.

Stimulation by 5FU was almost completely prevented by adding uracil at a concentration of 20 γ/ml. On the other hand, inclusion or omission of thymidine at the same concentration made no detectable difference. This indicates that the stimulating effect of 5FU is not due to its interference[21] with DNA synthesis.

It is essential to stress that the effect of 5FU is a physiological one and not due to mutagenesis. The progeny phage yielded by K in the presence of 5FU were no more active (in the absence of 5FU) than their parents.

Kinetics of the 5FU effect: To localize the time of action of 5FU in phage-infected cells, 3-minute pulse exposures to the analogue were given at various times before and after infection. The results (Fig. 3) show that pre-infection exposure causes little effect and that 5FU is most effective if present during the first 18 minutes at 26°. This is the first third of the eclipse period, a time during which there is active synthesis[1, 2] of messenger-RNA but before DNA synthesis has begun.[38]

That 5FU is rapidly incorporated into messenger-RNA has been shown by Gros *et al.*[22] Their studies were on uninfected cells, but, as indicated by the work of Nomura, Hall, and Spiegelman,[10] messenger-RNA may in fact be the only kind of RNA made after phage infection.

As Garen[23] and Nomura[24] have shown, the metabolism of an *r*II-infected K cell is

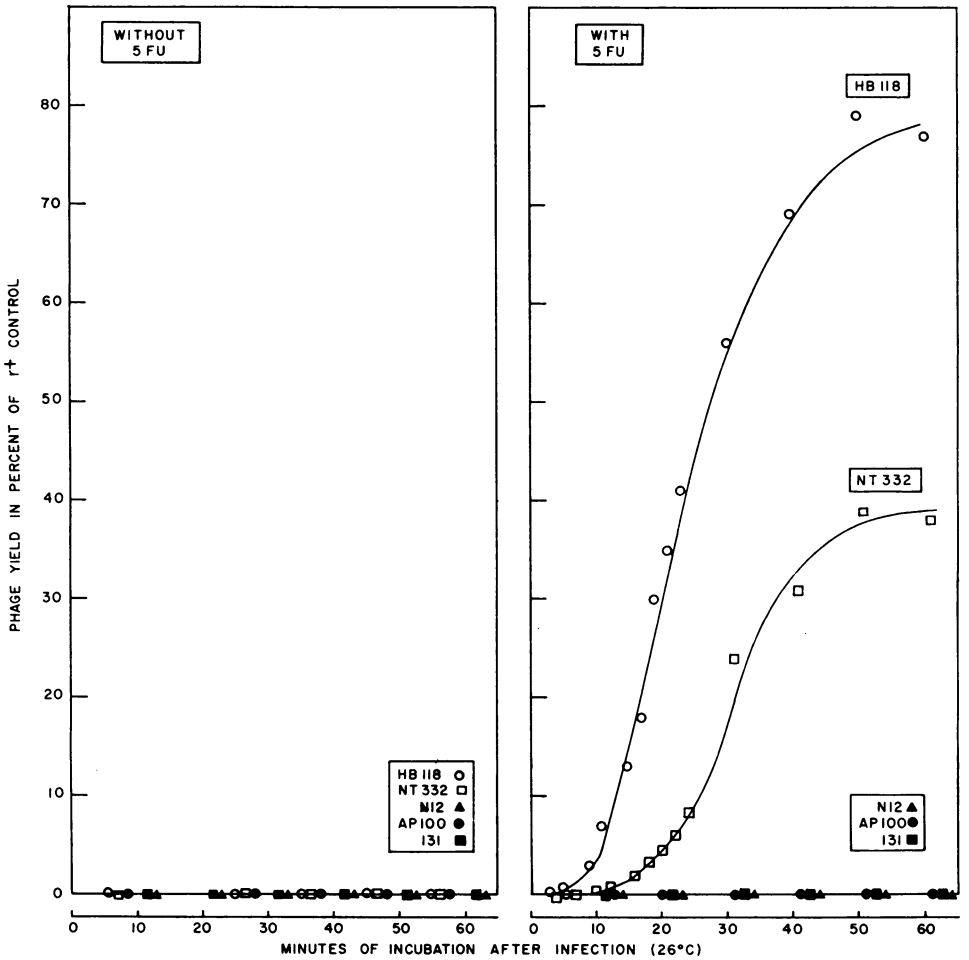

FIG. 2.—Effect of 5-fluorouracil on the activity of various *r*II mutants in *E. coli* K.
To K growing exponentially in M9S at 2.3×10^8 cells/ml, 0.01 *M* NaCN was added followed by phage at 0.3 particle per bacterium. After 10 min adsorption at 37°C, anti-T4 serum was added to eliminate unadsorbed phage. Ten min later, phage development was initiated (time zero on the graph) by diluting 500-fold into M9S + 20 γ/ml thymidine at 26°C either without or with 10 γ/ml 5FU. At various times thereafter, samples were diluted 50-fold into broth supplemented with 20 γ/ml of uracil and 20 γ/ml of thymidine. After one hour at 37°C, the broth tubes were shaken with a few drops of chloroform to complete lysis and assayed for phage on *E. coli B*. As a control, r^+ phage (not shown) was measured in precisely the same way. The r^+ yield decreased somewhat with progressively later shifts from synthetic medium to broth medium so that the data for the mutants are expressed as per cent of the yield for r^+.

at first normal and even proceeds as far as the production of DNA replicas before the cell ceases to function. Nevertheless, standard type recombinants arising during replication are unable to remove the block.[25] This decision is presumably dictated by messenger-RNA made directly from the DNA of the infecting phage particles.

Generality of the 5FU effect: If the mechanism postulated for the action of 5FU on *r*II mutants is correct, the same phenomenon should be observable with mutants affected in other cistrons. This has indeed been found for mutants of *E. coli*

strain K10 that are defective in the enzyme alkaline phosphatase. Twenty-five phosphatase-negative mutants,[12, 13] generously supplied by Dr. Alan Garen, were tested. Each strain was grown in high phosphate medium (to repress formation of any enzyme) and then shifted to low phosphate medium, in which condition formation of the enzyme (if any) is induced.[15] When induction was done in the presence of 5FU, two of the mutants formed active enzyme. Figure 4 compares a responsive mutant (U8) with a nonresponsive mutant (U12). Thus, the 5FU effect, although highly site-specific within a given cistron, is of general applicability to other cistrons.

The increase in enzyme activity of U8 induced in the presence of 5FU, while some 30-fold over the control, was small (about 0.5%) relative to the phosphatase-positive strain under the same conditions. In the case of the *r*II mutants shown in Figure 2, the phage yield may, of course, be far out of proportion to the amount of r^+ activity.

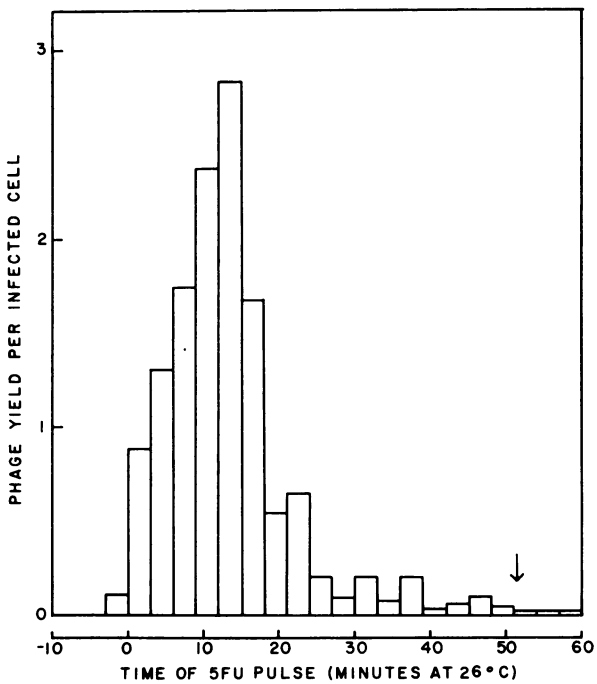

FIG. 3.—Effect of a three-minute pulse of 5-fluorouracil, applied at various times, on *r*HB118 infecting *E. coli* K. The procedure was as in Fig. 2 except that the infected cells were allowed to develop in M9S medium for various times before exposure to 5FU. The pulse was applied by diluting into an equal volume of the same medium plus 20 γ-ml 5FU. Three minutes later, the pulse was ended by dilution into uracil-containing broth and the experiment continued as in Fig. 2. Due to the short exposure to the analogue the phage yield per infected cell was much smaller than in Fig. 2. The arrow at 52 minutes indicates the end of the "eclipse" period, i.e., the time at which the number of mature phage particles reaches, on the average, one per cell for cells infected with r^+ in the absence of 5FU.

Identification of DNA base pairs by mutagens: When DNA replicates in the presence of analogues of the normal DNA bases, mutations are readily induced,[26] apparently due to errors in pairing which lead to the permanent substitution of one base pair for another. As argued by Freese,[27] such errors in base pairing should lead to the substitution of a purine for a purine and a pyrimidine for a pyrimidine without changing their orientation with respect to the two DNA chains (transitions). Two base analogues that have been studied extensively are 2-aminopurine (2AP) and 5-bromodeoxyuridine (BDU). These induce mutations specifically at certain genetic sites,[28, 27, 14] and the mutants are, as a rule, also inducible by base analogues to revert to standard type.[11]

From the finding that mutants induced by BDU tend to be strongly revertible by 2AP, and vice versa, Freese[11] suggested that one of the mutagens favored the transition GC → AT while the other induced AT → GC. An indication of which

is which came from experiments with hydroxylamine.[17, 29] Cytosine and its derivatives were shown to react more readily than the other bases that occur in DNA.

It is difficult, however, to extrapolate with assurance from the gross chemical effects of mutagens to the actual mutational events, which could be caused by a minority reaction. Schuster[30] and Brown and Schell[31] have shown that hydroxylamine indeed reacts to a slight extent with thymine in DNA. However, Schuster also showed that the rate of reaction of hydroxylamine with cytosine (in RNA) decreases with higher pH, whereas the reaction with uracil increases. The pH dependences of the reactions with thymine and hydroxymethylcytosine in phage DNA, although not yet determined, might be expected to be analogous to those of uracil and cytosine, respectively. To obtain further evidence as to which reaction is responsible for the mutagenic effect, we have examined the pH dependence of the mutagenic effect of hydroxylamine on the *r*II mutant *r*AP275. The observed mutation rates (induced revertants per survivor per unit time) at pH values of 6, 7.5, and 9 were in the ratio 28:12:1. The rates found by Schuster for the reaction with cytosine at similar pH values were in the ratio of 32:13:<4, whereas uracil reacted in the ratio <1:13:30. Six other mutants were tested and all showed the same pH dependence as *r* AP275.

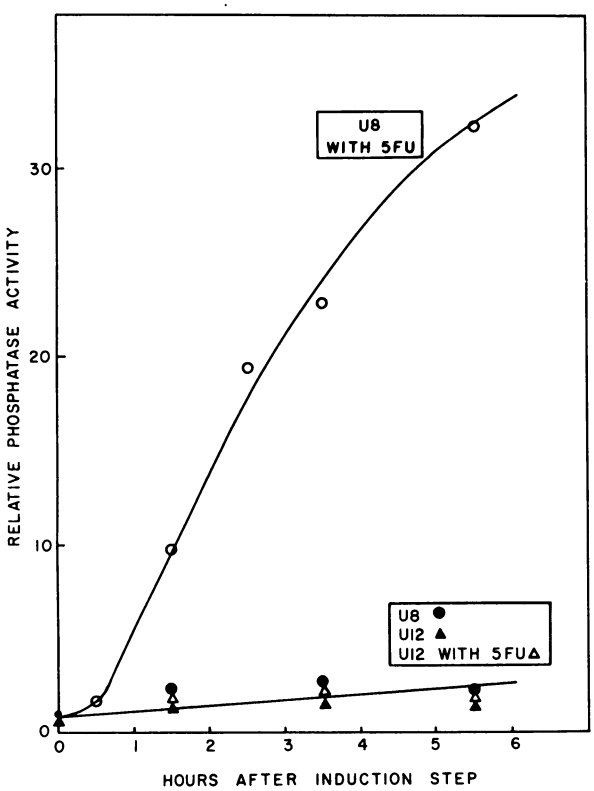

FIG. 4.—Reversal of a phosphatase-negative defect by 5-fluorouracil. Overnight cultures of the bacteria grown in repressing medium ("high phosphate" Tris-glucose) were diluted 20-fold into the same medium and aerated at 37°C for 3 hours. The cells were centrifuged, resuspended in chilled medium (less glucose and phosphate), centrifuged again, and resuspended in inducing medium ("low phosphate" Tris-glucose). The cultures were then divided into 2 parts, to one of which was added 20 γ/ml of 5FU, and both were aerated at 37°C (starting at time zero on the graph). Samples were taken at various times and assayed for alkaline phosphatase activity as described in *Methods*. The ordinate is in arbitrary units relative to the activity of mutant U8 at zero time.

This result reinforces the conclusion that hydroxylamine predominantly induces the transition GC → AT, and the correlation between hydroxylamine and BDU (Table 1, below) supports the conclusion that BDU acts in the same way. The action of 2AP, on the other hand, seems less specific than originally supposed. In the data of Freese *et al.*,[17] as well as those given below, 2AP-induced mutants are

about equally divided with respect to their revertibility by hydroxylamine. 2AP apparently induces both transitions with comparable ease.

Therefore, the following rules may be used for identification of base pairs. Given a mutant (of any origin), if it is inducible to revert by 2AP, it can be classed as a transition mutant. If it reverts strongly in response to BDU and hydroxylamine as well, this suggests that the mutant base pair is GC; if it does not, the mutant base pair indicated is AT. These criteria identify the base pair at the corresponding site in the standard type phage as AT in the former case and GC in the latter case.

*r*II mutations have been located at some hundreds of distinct sites in the two *r*II cistrons. Most of the sites have many recurrences, some spontaneous, some induced with one mutagen or another.[14] One representative of each site in the *r*II region was chosen (usually the first mutant to indicate the site) and tested quantitatively for induction of reversions by 2AP and BDU. Where an increase over the spontaneous rate was observed, the revertants were examined for plaque type on strain B (see *Methods*).

Of the 339 mutants tested, 69 were unambiguously inducible to revert by 2AP (and/or BDU) to a form which was judged to be phenotypically r^+ on strain B. (Some of these also showed false revertants in smaller numbers.) The mutants are listed in Table 1 (Groups I, II, and III) along with 40 mutants (Group IV) chosen at random from the larger group which showed no reversion induction. All 109 mutants were further tested for reversion by hydroxylamine.

Mutants of Groups I and II are those that are reverted strongly by 2AP but weakly, or not at all, by BDU. According to the rules based upon the mutagenic specificity of the analogues, these mutants must have arisen by GC → AT transitions. The base pairs deduced to be in the standard type DNA at the corresponding sites are listed in the table as GC.

Group III consists of mutants that respond not only to 2AP but also to BDU. It is seen that there is a good correlation between hydroxylamine and BDU induction, consistent with the idea that both of these agents act predominantly on GC base pairs. For mutants responding to *both* BDU and hydroxylamine, the base pair indicated in the standard type is AT. In the few cases where a mutant responds to BDU and not hydroxylamine, or vice versa, identification of the base pair cannot be made with assurance. The mutants of Group IV, not revertible by base analogues, could be transversions,[11] i.e., substitutions in which the orientation of purine and pyrimidine has been reversed, or tiny deletions or insertions.[32] For these mutants, no conclusion as to the original base pair can be reached.

Figure 5 shows the positions of the various mutations within the two cistrons of the *r*II region, as determined by genetic mapping. Mutations which showed no induced reversions by 2AP or BDU are represented as open circles. Those represented as solid circles are inducible to revert (either truly or falsely) by one or more of the mutagens. Where the base pair can be labeled unambiguously according to the data of Table 1, it is shown in the figure.

The remainder of the mutations indicated as responsive to mutagens showed either feeble induction or a large component of manifestly false revertants, and therefore no base pair designation can be given without further study. Of course, a revertant can be demonstrated to be false, but cannot be proved, by genetic analysis alone, to be identical with the original standard type, so that the possibility is not

TABLE 1

RESPONSE OF rII MUTANTS TO MUTAGENS AND TO 5FU

	Reverse Mutation Induction by Mutagens Reversion Index (K/B) in Units of 10^{-7}					Response to 5FU Progeny per cell		Summary				Indicated Bases r^+	
r^+	Spont.	2AP	BDU	NH_2OH Control	NH_2OH Treated	Control 126.	5FU 79.	2AP	BDU	NH_2OH	5FU	Phage DNA	Messenger-RNA
						GROUP I							
rN55	1.4	1700	2.8	0.	0.5	0.00	0.9	+	0	0	+	GC	C
rHB118	0.5	2300	0.	0.5	0.	0.00	56.	+	0	0	+	GC	C
rC204	0.0	730	0.2	0.	0.	0.00	1.3	+	0	0	+	GC	C
rN11	1.3	2800	5.3	0.	0.	0.00	1.5	+	0	0	+	GC	C
rEM64	0.4	2500	1.3	0.9	0.5	0.00	33.	+	0	0	+	GC	C
rHB35	0.1	1400	0.2	2.2	2.0	0.00	9.6	+	0	0	+	GC	C
rHB129	0.2	1000	0.3	1.6	0.	0.00	24.	+	0	0	+	GC	C
rHB84	0.0	750	0.1	0.	0.	0.00	14.	+	0	0	+	GC	C
rN21	0.0	450	0.0	0.	0.	0.01	29.	+	0	0	+	GC	C
rEM84	0.2	1600	0.6	0.4	3.1	0.00	5.2	+	0	0	+	GC	C
rN24	3.0	4600	5.9	8.6	20.	0.02	8.2	+	0	0	+	GC	C
rHB74	0.3	3000	0.5	0.8	3.	0.00	0.6	+	0	0	+	GC	C
rNT332	0.2	1400	1.6	1.9	1.6	0.00	22.	+	0	0	+	GC	C
rB94	0.0	2800	0.3	2.	5.	0.00	7.4	+	0	0	+	GC	C
rSD160	0.5	1500	3.6	0.	3.	0.01	2.8	+	0	0	+	GC	C
rHB232	0.1	300	0.5	0.9	2.5	0.02	11.	+	0	0	+	GC	C
rAP53	0.6	890	0.5	0.3	2.5	0.04	48.	+	0	0	+	GC	C
						GROUP II							
rNA27	0.1	1600	0.5	1.	3.	0.00	0.05	+	0	0	0	GC	G
rN74	0.0	390	0.5	0.5	0.	0.04	0.2	+	0	0	0	GC	G
r1274	0.0	330	7.0	2.8	3.5	0.00	0.00	+	0	0	0	GC	G
r1249	0.1	1600	20.	1.8	1.7	0.00	0.01	+	0	0	0	GC	G
rNB4777	0.6	630	58.	2.4	4.8	0.00	0.05	+	0?	0	0	GC	G
rHB122	0.1	14000	4.6	0.7	0.5	0.00	0.08	+	0	0	0	GC	G
rBC11	0.0	15000	0.	1.	0.5	0.00	0.06	+	0	0	0	GC	G
rUV47	0.2	280	9.	0.9	0.8	0.00	0.05	+	0	0	0	GC	G
rAP211	0.0	200	0.5	0.	1.2	0.00	0.06	+	0	0	0	GC	G
rUV1	0.0	350	0.1	0.	0.	0.00	0.03	+	0	0	0	GC	G
r425	0.3	3300	1.4	0.	0.	0.00	0.00	+	0	0	0	GC	G
rEM20	0.0	3000	0.8	1.	0.5	0.00	0.01	+	0	0	0	GC	G
rUV122	0.5	1600	18.	2.2	3.7	0.16	0.06	+	0	0	0	GC	G
rHB32	0.1	4800	1.8	1.8	2.7	0.00	0.09	+	0	0	0	GC	G
r585	0.0	330	0.7	0.	5.	0.00	0.01	+	0	0	0	GC	G
r1310	0.0	1000	0.0	0.	0.5	0.00	0.00	+	0	0	0	GC	G
rHB80	0.0	3500	12.	0.4	0.8	0.00	0.07	+	0	0	0	GC	G
rAP126	0.0	2900	0.7	0.	0.8	0.00	0.06	+	0	0	0	GC	G
rUV375	0.6	6700	24.	0.9	1.7	0.00	0.04	+	0	0	0	GC	G
r360	0.0	530	0.6	0.	0.	0.00	0.03	+	0	0	0	GC	G
r375	0.3	2200	2.0	0.8	1.1	0.00	0.00	+	0	0	0	GC	G
rN17	0.0	1500	0.4	0.	0.	0.00	0.09	+	0	0	0	GC	G
rN90	0.4	8600	4.5	1.	2.	0.01	0.06	+	0	0	0	GC	G
rUV199	2.4	5900	7.1	0.7	0.3	0.00	0.2	+	0	0	0	GC	G
rN12	0.2	8500	1.3	0.6	1.5	0.02	0.05	+	0	0	0	GC	G
rN29	0.0	7600	0.	0.	4.	0.00	0.00	+	0	0	0	GC	G
rAP61	1.2	500	2.9	1.9	4.1	0.02	0.1	+	0	0	0	GC	G
r979	1.5	3200	24.	5.7	7.1	0.01	0.08	+	0	0	0	GC	G
rEM7	0.1	2000	0.6	0.2	0.0	0.00	0.04	+	0	0	0	GC	G
r1814	1.	8200	10.	0.9	72.	0.04	0.2	+	0	+	0	..	..
r287	2.1	2800	1.0	6.4	150.	0.00	0.00	+	0	+	0	..	..

The mutants are divided into groups according to their responses to mutagens and to 5FU. Within each group, they are listed according to their order in the recombination map (Fig. 5). In cases of positive response to 2AP or BDU, the reversion test was done two or more times and the value listed is the average reversion index, excluding extreme values occasionally observed due to "jackpots." A zero in the last decimal place indicates that the value in that place is less than one.

The control for hydroxylamine reversion is in some cases different from the spontaneous reversion index due to fluctuations of the r^+ background from one stock to another. Procedures for the mutagen tests are described in *Methods*. The measurements of response to 5FU were made as described in Figure 2, the dilution from the test medium being made about 45 minutes after the initiation of phage development.

ruled out that the apparent specificity is incorrect in certain cases. A more stringent test of genuineness is to cross the presumed revertant to standard type, in which case a suppressor mutation at another site may be revealed by the appearance of the unsuppressed mutant as a recombinant. A still more stringent test is to show that the revertant has the same forward mutability, at the same site, as

TABLE 1 (Continued)

RESPONSE OF rII MUTANTS TO MUTAGENS AND TO 5FU

	Reverse Mutation Induction by Mutagens Reversion Index (K/B) in Units of 10^{-7}					Response to 5FU Progeny per cell		Summary				Indicated Bases	
	Spont.	2AP	BDU	NH_2OH Control	NH_2OH Treated	Control	5FU	2AP	BDU	NH_2OH	5FU	r^+ Phage DNA	r^+ Messenger-RNA
GROUP III													
rAP129	4.1	6200	2100.	4.	680.	0.1	0.4	+	+	+	0	AT	..
rAP218	2.6	1200	250.	1.	730.	0.01	0.2	+	+	+	0	AT	..
rH221	0.4	460	220.	0.	26.	0.00	0.04	+	+	+?	0	AT	..
rAP100	2.0	80	1800.	3.	2600.	0.00	0.01	+	+	+	0	AT	..
r607	0.9	340	7100.	1.9	1500.	0.03	0.02	+	+	+	0	AT	..
rEM114	30.	3200	1200.	100.	890.	0.5	0.8	+	+	+	0	AT	..
rNT88	0.5	230	500.	0.1	160.	0.00	0.04	+	+	+	0	AT	..
rDAP56	3.6	5500	2000.	16.	3100.	0.00	0.01	+	+	+	0	AT	..
r380	0.5	6600	2400.	0.	220.	0.4	1.8	+	+	+	+?	AT	..
rSN103	0.9	580	1800.	2.2	370.	0.01	0.07	+	+	+	0	AT	..
r263	7.8	3700	8900.	27.	3600.	0.6	3.3	+	+	+	+?	AT	..
rF72	0.7	120	680.	2.6	770.	0.00	0.01	+	+	+	0	AT	..
rJ33	1.5	2000	890.	4.	230.	0.00	0.04	+	+	+	0	AT	..
rBC35	2.1	46	760.	1.2	660.	0.00	0.01	+	+	+	0	AT	..
rAP275	1.9	580	4500.	4.1	1600.	0.00	0.04	+	+	+	0	AT	..
rUV363	0.7	26	3000.	3.5	600.	0.00	0.03	+	+	+	0	AT	..
rUV181	0.07	940	470.	0.8	1.4	0.00	0.06	+	+	0	0	..	..
rG178	0.07	970	86.	0.	0.5	0.00	0.02	+	+	0	0	..	..
r2074	4.7	25000	390.	19.	16.	0.00	0.01	+	+	0	0	..	..
r609	3.3	8800	450.	3.0	1.0	0.02	0.04	+	+	0	0	..	..
r1221	1.9	1900	170.	9.2	14.	0.2	5.6	+	+	0	+	..	..
GROUP IV													
r681	3.1	1.7	3.4	8.	16.	0.00	0.00	0	0	0	0	..	..
rUV11	0.1	0.1	0.4	0.	0.	0.00	0.02	0	0	0	0	..	..
r569	0.9	1.3	4.7	0.5	0.6	0.02	0.1	0	0	0	0	..	..
r1176	0.2	0.0	0.2	0.	0.	0.00	0.00	0	0	0	0	..	..
rUV68	1.2	1.1	3.0	2.6	1.4	0.00	0.01	0	0	0	0	..	..
rNT311	0.0	0.1	0.0	0.9	0.8	0.00	0.00	0	0	0	0	..	..
rBC81	0.0	0.0	0.1	0.0	0.0	0.00	0.01	0	0	0	0	..	..
r227	0.1	0.0	0.	0.	0.	0.00	0.00	0	0	0	0	..	..
r577	0.1	2.2	0.0	0.	0.	0.00	0.00	0	0	0	0	..	..
r1084	0.4	2.5	2.2	1.	0.	0.00	0.00	0	0	0	0	..	..
r205	0.0	0.0	0.1	0.5	0.5	0.00	0.01	0	0	0	0	..	..
rH201	3.5	0.8	3.5	0.8	2.9	0.00	0.00	0	0	0	0	..	..
r465	1.7	3.0	4.2	7.6	6.5	0.15	0.04	0	0	0	0	..	..
r1470	0.2	0.2	0.2	0.	0.	0.00	0.01	0	0	0	0	..	..
r447	0.4	0.3	0.2	0.8	1.4	0.00	0.01	0	0	0	0	..	..
rDAP66	1.0	2.0	1.3	0.7	1.	0.00	0.00	0	0	0	0	..	..
rH51	0.2	0.3	0.5	0.5	0.3	0.00	0.02	0	0	0	0	..	..
r2232	1.8	1.9	1.9	4.	2.	0.00	0.02	0	0	0	0	..	..
rP5	2.2	8.1	3.7	6.	5.	0.00	0.05	0	0	0	0	..	..
rF27	4.1	1.0	5.3	1.2	27.	0.00	0.00	0	0	+?	0	..	..
r285	0.6	0.3	0.4	0.6	3.3	0.00	0.00	0	0	0	0	..	..
rC135	0.2	0.2	0.2	0.	0.	0.00	0.01	0	0	0	0	..	..
rSN181	2.4	0.7	2.2	10.	8.	0.01	0.02	0	0	0	0	..	..
rD2	3.9	4.7	4.0	3.5	43.	0.00	0.02	0	0	+?	0	..	..
r173	0.0	0.0	0.0	0.	0.	0.01	0.02	0	0	0	0	..	..
rUV118	0.3	0.2	0.4	0.7	0.	0.00	0.03	0	0	0	0	..	..
r240	0.1	0.0	0.1	3.6	3.7	0.00	0.01	0	0	0	0	..	..
r131	2.0	3.7	6.6	4.1	3.0	0.01	0.03	0	0	0	0	..	..
r244	8.0	6.1	22.	13.	14.	0.00	0.01	0	0	0	0	..	..
r326	0.3	0.6	1.2	0.	0.	0.00	0.01	0	0	0	0	..	..
rP4	0.3	0.0	0.	0.1	0.	0.00	0.00	0	0	0	0	..	..
rAP176	0.1	0.2	0.0	1.8	1.7	0.00	0.02	0	0	0	0	..	..
r117	16.	2.7	5.0	27.	37.	0.00	0.00	0	0	0	0	..	..
r1467	6.3	5.7	3.4	8.9	7.5	0.00	0.01	0	0	0	0	..	..
rB13	0.0	0.1	0.2	0.3	0.0	0.00	0.00	0	0	0	0	..	..
rJ241	0.1	0.1	0.7	0.	0.7	0.00	0.01	0	0	0	0	..	..
rEM113	0.0	0.1	0.1	0.	0.	0.00	0.01	0	0	0	0	..	..
rEM29	0.0	0.3	0.0	0.	0.	0.00	0.01	0	0	0	0	..	..
rUV124	0.0	0.0	0.0	0.5	0.0	0.00	0.02	0	0	0	0	..	..
rEM87	0.0	0.0	0.	0.5	0.6	0.00	0.01	0	0	0	0	..	..

does the standard type. While both of these criteria are satisfied by some revertants that have been studied, the tests have not yet been applied to the various mutants of Table 1. It is possible also that the 5FU effect could, in certain instances, act at a site distant from the one in question, producing the phenotypic equivalent of a suppressor mutation.

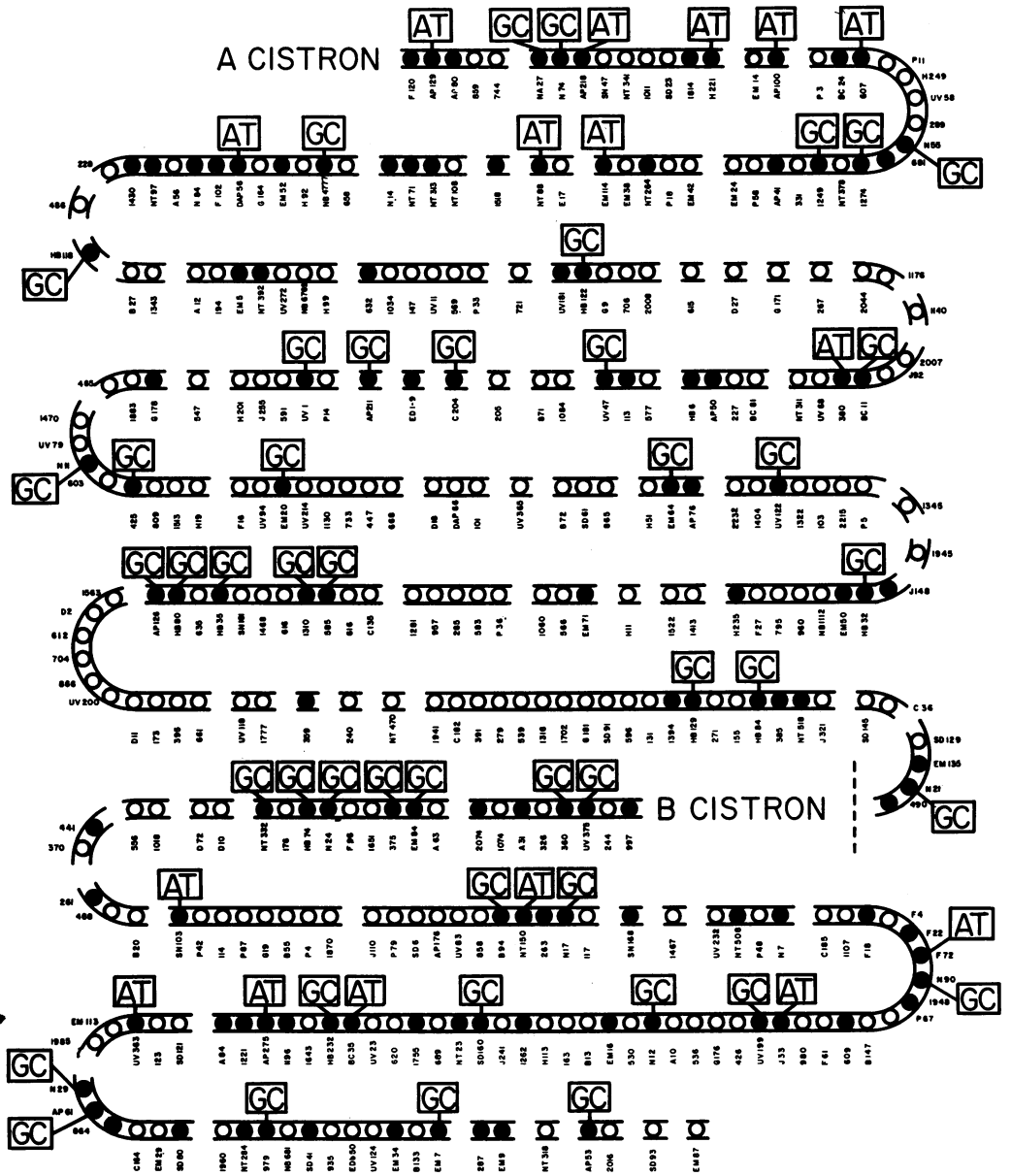

FIG. 5.—Genetic map of the *r*II region showing the base pairs in the standard type as deduced from the data of Table 1. Each circle represents a distinct mutational site. Breaks in the map indicate segments as defined by the ends of deletions. While the order of the segments is known, the arrangement of sites within any one segment has not been determined.

● Indicates mutants which are inducible to revert by one or more of the mutagens tested (2AP, BDU, or hydroxylamine). Where reversion was weak or false, no base pair is assigned.

○ Indicates mutants for which no induced reversion was detected with 2AP or BDU.

Specificity of response to 5FU: All of the mutants in Table 1 have been tested for reversal of phenotype by 5FU and the results are listed.

The first expectation from the proposed mechanism, namely, that only transition

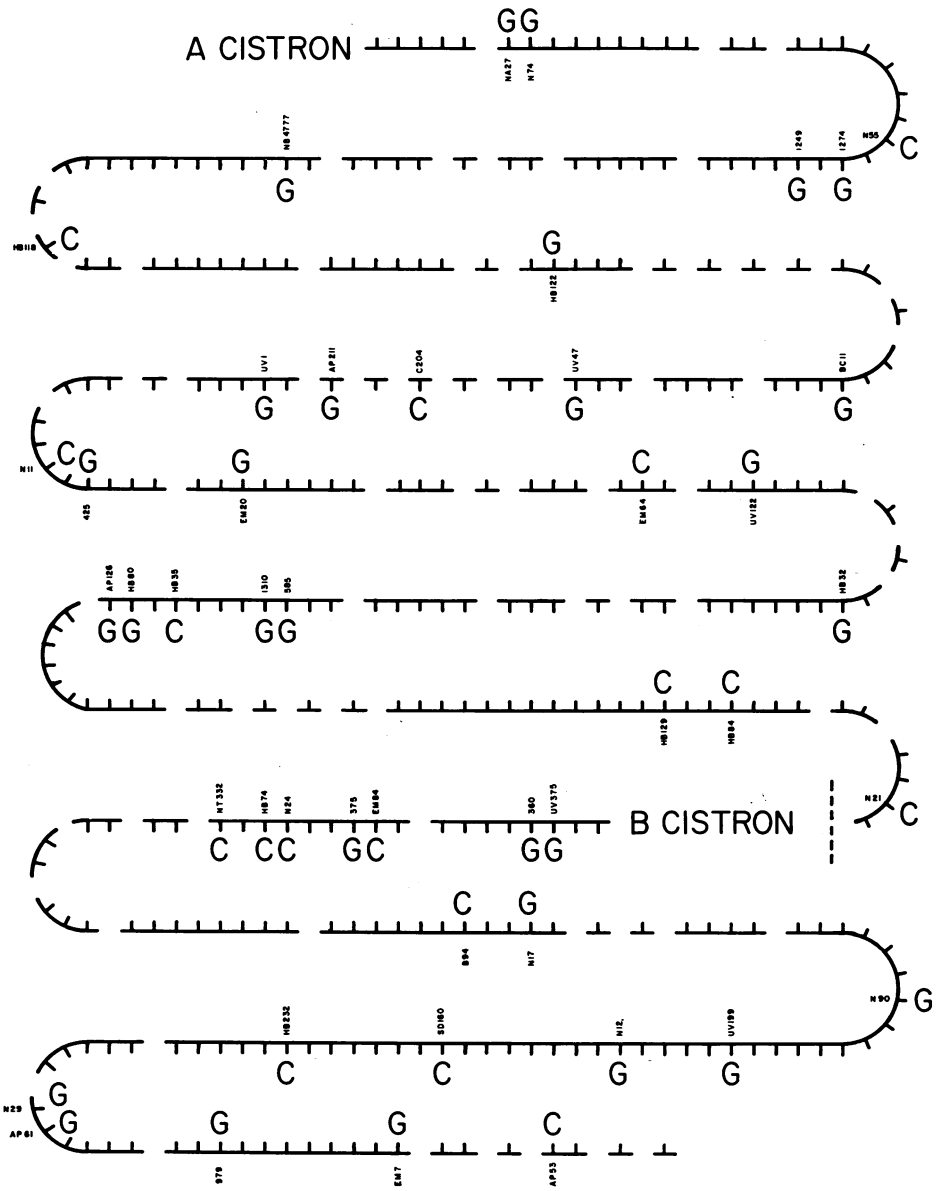

FIG. 6.—Map of the messenger-RNA for the *r*II region showing the nucleotides (for the standard type phage) suggested by the data of Table 1 (Groups I and II).

mutations (Groups I, II, and III) should respond to 5FU, is seen to be rigorously fulfilled. Out of the 20 mutants judged to have a positive response to 5FU, 17 are among those induced to revert only by 2AP. The other three (having rather high control values and weak response) are induced to revert also by BDU. This distribution implies that 5FU acts predominantly by being incorporated into messenger-RNA at sites corresponding to AT in the DNA. That is, it enters in place of U and acts partially like C. The weak but reproducible response observed with several

BDU-reverting mutants is not surprising if 5FU can also (but more rarely) be incorporated in place of C.

Unfortunately there is not an absolutely sharp separation between 5FU responders and nonresponders. A slight though variable response to 5FU can be detected for almost every mutant. This small nonspecific effect can be seen for some of the Group IV nontransition mutants, and occurs even with deletion mutants, so it would seem to be due to a partial undoing of the general block against *r*II mutants. Even for mutants that definitely respond, the degree of response varies. This is perhaps ascribable to the influence of neighboring bases on the efficiency of incorporating the analogue or on its properties once it is in.

From Table 1 (Groups I and II) it is evident, nevertheless, that the mutants revertible solely by 2AP include some (17) that are responsive to 5FU and others (29) that are not. This is close to the expectation if only one DNA strand is copied as a useful messenger. Response to 5FU would thus specify the relative orientation of the DNA base pairs at the sites in question. Given a GC base pair in the standard type, which becomes, in the mutant, AT, the latter will respond to 5FU only if the A is in the "major" strand of the DNA, i.e., the one which is transcribed into messenger-RNA. Thus, a 5FU-responsive mutant would be one which has G at the corresponding site in the major strand of the DNA of the standard type. It follows that the base in the standard type messenger-RNA is C. Figure 6 summarizes the various nucleotides in normal *r*II messenger-RNA deduced according to this scheme.

Discussion.—Unlike the analogues BDU and 2AP, which cause heritable changes in the DNA, 5FU causes a temporary change in phenotype. It is effective on phage only if added in the period shortly after infection during a time when messenger-RNA is actively synthesized but soluble RNA and ribosomal RNA are not. Mutants that are not induced to revert by base analogues do not respond to 5FU. Of those mutants identified by chemical mutagenesis as having an AT pair at the mutant site, somewhat less than half respond more or less strongly to 5FU. Conversely, among mutants identified as containing GC pairs, response to 5FU is uncommon.

These observations are consistent with the scheme presented in Figure 1. The fact that roughly half of the AT mutants respond to 5FU would appear to suggest that only one strand of the DNA is transcribed as functional messenger-RNA. The 5FU effect makes it possible to define the orientation of the base pair in the two DNA strands and thus provides the key needed to reduce the genetic map to nucleotide sequences along one of the DNA strands. The messenger-RNA would then have the complementary sequence. 5FU permits the orientation of only one of the two kinds of DNA base pairs. It would obviously be desirable to find an analogue of cytosine or guanine, which would serve for the other.

The results suggest that only one *physiologically active* messenger is made from the two-stranded DNA, as also suggested by certain genetic experiments of Tessman.[33] However, it is known from the work of Chamberlin and Berg[34] that, *in vitro*, both strands of DNA *can* be copied as RNA by ribonucleotide polymerase. It may be, nevertheless, that for an intact chromosome, *in vitro*, there is a mechanism fixing the direction in which the messenger copies the DNA. Suggestive evidence for one-strand copying comes from the experiments of Bautz and Hall,[35] who

have isolated T4 messenger-RNA and measured its base composition. Their results reveal a deviation from identity between the base compositions of T4 DNA and the messenger-RNA. This indicates that messenger-RNA copies only one DNA strand, and further that the bases are not equally distributed between the two strands. They found 21 per cent G and 15 per cent C in the purified T4 messenger-RNA. Although the number of G and C sites so far indicated for the *r*II messenger (Fig. 6) is not large enough to give a statistically reliable sampling and may also be a random sample of all possible G and C sites, it is interesting to note that they deviate from equality in the same direction, as observed by Bautz and Hall.

In the set of mutants studied so far, ones arising from changes at GC sites are about twice as numerous as those arising at AT sites. This might appear to be in contradiction with the known composition of T4 DNA, in which the relative abundance of the base pairs is just the reverse. However, this may very easily be due to the fact that most of the mutagens so far used in producing the mutants turn out to act preferentially on GC pairs. If this is so, it should prove possible to detect many more sites by exhaustive mapping of mutants induced by 2AP.

Regarding the behavior of 5FU in base pairing, Lengyel *et al.*[36] found polyfluorouridylic acid to be rather ineffective in stimulation of polyphenylalanine synthesis (as compared to polyuridylic acid, which is extremely effective).[37] This is evidence that the fluorine atom does cause an alteration in the behavior of U. By studying the properties of a mixed polymer made of U and 5FU, it should be feasible to determine directly whether 5FU indeed acts partially like C in coding for amino acids.

Summary.—5-Flourouracil partially reverses the defective phenotypes of certain *r*II mutants of phage T4 (as well as certain phosphatase-negative mutants of *E. coli*). From the kinetics of the effect and the specificity of action on various *r*II mutants, taken together with the responses of the mutants to specific mutagens, it is concluded that 5FU acts mainly by incorporation into messenger-RNA in place of uracil, there acting partially like cytosine. The results are consistent with the idea that only one strand of the DNA duplex is copied into useful messenger-RNA. This provides a means for determining the orientation of the nucleotide pairs with respect to the two DNA chains, as well as identification of the nucleotides at the corresponding sites in the *r*II messenger-RNA.

We wish to express our gratitude to Mmes. Daine Auzins, Judith Berry, and Barbara Williams for their tireless and able assistance with the experiments. This research has been supported by grants from the National Science Foundation and the National Institutes of Health.

[1] Volkin, E., and L. Astrachan, *Virology*, **2**, 149 (1956).
[2] Astrachan, L., and E. Volkin, *Biochim. et Biophys. Acta*, **29**, 536 (1958).
[3] Jacob, F., and J. Monod, *J. Mol. Biol.*, **3**, 318 (1961).
[4] Brenner, S., F. Jacob, and M. Meselson, *Nature*, **190**, 576 (1961).
[5] Gros, F., H. Hiatt, W. Gilbert, C. G. Kurland, R. W. Risebrough, and J. D. Watson, *Nature*, **190**, 581 (1961).
[6] Horowitz, J., and E. Chargaff, *Nature*, **184**, 1213 (1959).
[7] Naono, S., and F. Gros, *C.R. Acad. Sci. (Paris)*, **250**, 3889 (1960).
[8] Bussard, A., S. Naono, F. Gros, and J. Monod, *C.R. Acad. Sci. (Paris)*, **250**, 4049 (1960).
[9] Benzer, S., and S. P. Champe, these PROCEEDINGS, **47**, 1025 (1961).
[10] Nomura, M., B. D. Hall, and S. Spiegelman, *J. Mol. Biol.*, **2**, 306 (1960).
[11] Freese, E., *J. Mol. Biol.*, **1**, 87 (1959).

[12] Levinthal, C., in *Structure and Function of Genetic Elements*, Brookhaven Symposia in Biology, **12**, 76 (1959).

[13] Garen, A., in *Microbial Genetics*, ed. W. Hayes and R. C. Clowes (Cambridge University Press, 1960).

[14] Benzer, S., these PROCEEDINGS, **47**, 403 (1961).

[15] Torriani, A., *Biochim. et Biophys. Acta*, **38**, 460 (1960).

[16] Luria, S. E., and M. Delbrück, *Genetics*, **28**, 491 (1943).

[17] Freese, E., E. Bautz, and E. Bautz-Freese, these PROCEEDINGS, **47**, 845 (1961).

[18] Crick, F. H. C., L. Barnett, S. Brenner, and R. J. Watts-Tobin, *Nature*, **192**, 1227 (1961).

[19] Feynman, R., personal communication.

[20] Garen, A., and C. Levinthal, *Biochim. et Biophys. Acta*, **38**, 470 (1960).

[21] Cohen, S. S., J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, these PROCEEDINGS, **44**, 1004 (1958).

[22] Gros, F., W. Gilbert, H. H. Hiatt, G. Attardi, P. F. Spahr, and J. D. Watson, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (in press).

[23] Garen, A., *Virology*, **14**, 151 (1961).

[24] Nomura, M., *Virology*, **14**, 164 (1961).

[25] If K is infected with two *r*II mutants containing mutations far apart in the A cistron, r^+ recombinants should occur in many of the cells since DNA replicas are known to be formed. However, in such an experiment there is still no progeny yield such as would be expected if the recombinants could express their phenotype.

[26] Litman, R. M., and A. B. Pardee, *Nature*, **178**, 529 (1956).

[27] Freese, E., these PROCEEDINGS, **45**, 622 (1959).

[28] Benzer S., and E. Freese, these PROCEEDINGS, **44**, 112 (1958).

[29] Freese, E., E. Bautz-Freese, and E. Bautz, *J. Mol. Biol.*, **3**, 133 (1961).

[30] Schuster, H., *J. Mol. Biol.*, **3**, 447 (1961).

[31] Brown, D. M., and P. Schell, *J. Mol. Biol.*, **3**, 709 (1961).

[32] Brenner, S., L. Barnett, F. H. C. Crick, and A. Orgel, *J. Mol. Biol.*, **3**, 121 (1961).

[33] Tessman, I., in *The Molecular Basis of Neoplasia*, 15th Annual Symposium on Fundamental Cancer Research, Houston, in press.

[34] Chamberlin, M., and P. Berg, these PROCEEDINGS, **48**, 81 (1962).

[35] Bautz, E., and B. Hall, these PROCEEDINGS, **48**, 400 (1962).

[36] Lengyel, P., J. F. Speyer, and S. Ochoa, these PROCEEDINGS, **47**, 1936 (1961).

[37] Nirenberg, N. M., and J. H. Matthaei, these PROCEEDINGS, **47**, 1558 (1961).

[38] Cohen, S. S., *J. Biol. Chem.*, **174**, 218 (1948).

---

*EVIDENCE FOR THE PERSISTENCE IN PROTEIN SYNTHESIS OF AN INFORMATION TRANSFER MECHANISM AFTER THE REMOVAL OF GENES**

BY ALLEN S. FOX, SEI-BYUNG YOON, AND CHARLES G. MEAD†

DEPARTMENT OF BIOCHEMISTRY, MICHIGAN STATE UNIVERSITY, EAST LANSING

Communicated by M. R. Irwin, February 16, 1962

Recent reports have given evidence that enzyme synthesis in *E. coli* ceases immediately upon disruption of the genetic material involved,[1] apparently because of the instability of the RNA intermediate which carries information from genes to ribosomes in protein synthesis.[2] On the other hand, there is a rich literature